Remarks

The foregoing amendments are believed to place the claims into condition for immediate allowance or into better condition for consideration on appeal. 37 C.F.R. § 1.116(a). Accordingly, their entry after final rejection is respectfully respected.

Claims 33 and 35 are being cancelled herein and claims 14, 28-30, 32, 34, and 36 have been amended solely to expedite allowance. Support for the amendment to claims 28 and 29 can be found in the present specification at, *e.g.*, page 19, line 22, to page 20, line 1. Support for the amendment to claims 14, 30, 32 can be found in the present specification at, *e.g.*, page 16, lines 26-29 and page 27, lines10-20. The amendments to claims 34 and 36 merely adjust claim dependency, in view of the cancelled claims. Thus, these amendments are believed to introduce no new matter.

Upon entry of the foregoing amendments, claims 14-32, 34, and 36 are pending in the application, with claims 14 and 32 being the independent claims. Based on the above amendments and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding rejections and that they be withdrawn.

Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 14-32 remain rejected under 35 U.S.C. § 112, first paragraph, because, according to the Examiner, the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims. *See,* Paper No. 16, page 3. While the Examiner has indicated subject matter deemed enabled (Paper No. 16, page 2), the Examiner

maintains that the specification does not reasonably provide enablement for the claimed method wherein the vector is delivered "by any route of administration, particularly the use of any replication competent viral vectors and/or pathogenic live prokaryotic vectors through a systemic delivery, or the same method using any prokaryotic vector, particularly a prokaryotic expression plasmid vector, any pathogenic, non-attenuated live bacterial vector, or a mammalian artificial chromosome." Paper No. 16, page 3, lines 1-5. Applicants respectfully traverse this rejection.

The point of novelty of the presently claimed invention is that the expression of FPGS in neoplastic cells (at a level higher than the endogenous FPGS level), will enhance the cytotoxic sensitivity of the neoplastic cells to an antifolate drug, such as methotrexate or edatrexate. The particular type of gene delivery system that can deliver the FPGS gene includes viral vectors, non-viral vectors (including cellular vectors), or a hybrid of the two; *see*, specification, pages 15-20, and will, of course vary, based on numerous factors including, the particular tumor type, the tumor location, the condition of the patient, and the capabilities and/or particular expertise of a given laboratory, to name a few. These factors, as well as others, will be considered by those skilled in the art when determining the best suited gene delivery system.

The above notwithstanding, solely to expedite allowance, and without acquiescing to the propriety of the rejection, claims 14, 28-30, and 32 have been amended herein to accommodate the majority of the Examiner's concerns. In further support of attenuated prokaryotic vector technology, Applicants attach herein as Exhibit A, a copy of a publication by Bermudes *et al.*, "Live Bacteria as Anticancer Agents and Tumor Selective Protein Delivery," *Curr. Opin. Drug Discov. Devel.* 5:194-199 (2002). The authors of this paper

describe the use of several attenuated bacteria, such as *Salmonella*, *Clostridium*, and *Bifidobacterium* as tumor specific gene delivery vectors. They also report that an attenuated strain of *Salmonella typhimurium* is currently undergoing phase I clinical trials in cancer patients. *See*, Exhibit A, Abstract, last sentence, and page 8, reference citation no. 37.

With respect to the currently amended claims, Applicants submit that the reasonable practice of the claimed invention would not be substantially impeded due to the presence of "mammalian artificial chromosomes" within the scope of the claims. A rejection for lack of enablement on the basis of an inoperative embodiment is only proper when it is shown that a skilled artisan would require more than routine experimentation to distinguish the inoperative from the operative embodiments. *See, Atlas Powder Co. v. E.I. du Pont de Nemours & Co.*, 224 U.S.P.Q. 409 (Fed. Cir. 1984). The Examiner has not set forth any objective evidence that it would require undue experimentation to distinguish the operative from the supposed inoperative embodiments of the claimed invention. Accordingly, the presence of mammalian artificial chromosomes within the scope of the present claims does not provide a permissible basis of rejection under 35 U.S.C. § 112, first paragraph.

Accordingly, Applicants believe that this rejection under 35 U.S.C. § 112, first paragraph, has been overcome and should be withdrawn.

Rejections Under 35 U.S.C. § 103

The Examiner maintains the rejection of claims 14-27 and 30-35 under 35 U.S.C. § 103(a), because, according to the Examiner, these claims are unpatentable over Moscow *et al.*, U.S. Patent 5,763,216 ("Moscow"), in view of Roy *et al.*, *J. Biol. Chem. 272*: 6903-

6908 (1997) ("Roy"), Kim *et al.*, *J. Biol. Chem. 268*:21680-21685 (1993) ("Kim"), and Garrow *et al.*, *Proc. Nat'l. Acad. Sci. 89*:9151-9155 (1992) ("Garrow") for the reasons set forth previously in Paper No. 13. *See,* Paper No. 16, page 9, first paragraph.

In Paper No. 13, the Examiner stated:

> It would have been obvious and within the scope of skills for an ordinary skilled artisan to modify the method of Moscow *et al.* by direct delivery of a non-viral (plasmid) or viral vector comprising a DNA sequence encoding human FPGS into neoplastic cells *in vivo* that have acquired resistance to methotrexate and other classical folate analogues in order to reverse the resistance of MTX or other antifolate drugs in these neoplastic cells, so that to enhance the efficacy of conventional anti-folate drug therapy in light of the teachings of Roy *et al.*, Kim *et al.*, and Garrow *et al.* It is noted that as defined by the present application, a neoplastic cell is a cell whose normal growth control mechanism is disrupted thereby providing the potential for uncontrolled proliferation (citations omitted). As such, tumor cells resistant to MTX or other antifolate drugs would be encompassed within the scope of neoplastic cells of the instant invention. Furthermore, by reversing the resistance to MTX and other antifolate drugs in the tumor cells, the cytotoxic sensitivity of the tumor cells to an antifolate drug is in effects [sic] enhanced.

Paper No. 13, pages 23-24.

The Examiner also stated:

> One of ordinary [skill] in the art would have been motivated to carry out the above modification because Moscow *et al.*, Roy *et al.* and Kim *et al.* recognize <u>that decreased folylpolyglutamate synthetase is a factor contributing to</u> the resistance of tumor cells to methotrexate or other antifolate drug treatment, and by increasing the exogenous expression of FPGS in MTX or other antifolate resistant tumor cells, the sensitivity to antifolate drugs of the treated tumor cells would be enhanced and thereby enhancing the efficacy of traditional antifolate chemotherapy. (Emphasis in original).

Paper No. 13, page 24, lines 7-14.

Finally, the Examiner stated:

> One of an ordinary skilled artisan would have a reasonable expectation of success because Kim *et al.* clearly teach that lowered FPGS activity and decreased polyglutamylation of antifolates are thought to be general mechanisms by which cancer cells become resistant to a wide range of antifolates, and that FPGS-deficient mutant Chinese hamster ovary (CHO AUXB1) cells expressing high levels of human ... FPGS are more sensitive to the cytotoxicity of MTX compared to cells expressing lower levels of human FPGS (see Table III, page 21682). Furthermore, Roy *et al.*clearly show that L1210 tumor cells resistant to methotrexate or edatrexate have lowered FPGS activity. Therefore, the claimed invention as a whole was *prima facie* obvious in the absence of evidence to the contrary. (emphasis in original).

Paper No. 13, pages 24-25. Applicants respectfully traverse the rejection.

Applicants will now discuss the teachings of each reference individually, followed by a discussion concerning why one skilled in the art would not be motivated to make the substitutions necessary to arrive at the currently claimed invention.

The primary reference, the Moscow patent, relates solely to the human reduced folate carrier (RFC) gene, expression vectors comprising the RFC gene, as well as the use of such vectors to restore methotrexate (MTX) sensitivity to MTX-resistant, transport deficient, cancer cells. *See*, Moscow patent, Abstract. The MTX-resistant cancer cells studied in the Moscow patent contained markedly decreased expression of the RFC gene compared to the parental cell line. *Id.*, at sentence bridging cols. 15 and 16. According to the "Summary of the Invention" section of the Moscow patent, "The purpose of this [RFC] gene therapy is to restore RFC activity, and thereby re-establish the sensitivity of these cancer cells to MTX drug treatment." *Id.*, at col. 2, lines 6-12.

As discussed in the "Background of the Invention" section of the Moscow patent, RFC is one of two mechanisms by which animal cells uptake folate from their environment.

Accordingly, the RFC mechanism is involved in facilitating MTX (folate antagonist) uptake, and although other genes besides RFC may be involved in the development of MTX-resistance (including overexpression of dihydrofolate reductase, alteration of dihydrofolate reluctance affinity for MTX, decreased FPGS, and decreased thymidylate synthase levels), "decreased MTX uptake is the principal characteristic in many MTX-resistant cell-lines." Moscow Patent, col. 1, 2nd paragraph. Thus, a fair reading of the Moscow patent, as a whole, relates to: (1) the RFC gene (and not the FPGS gene), as the RFC gene is capable of facilitating MTX uptake; and (2) the restoration of RFC activity as a means to re-establish MTX sensitivity to MTX-resistant, transport deficient cancer cells.

Significantly, the Moscow patent teaches RFC gene transfer at the exclusion of all other known genes involved in folate metabolism (see list in prior paragraph), thereby "teaching away" from using other genes (such as FPGS) that may be involved in the acquisition of MTX resistance. As correctly acknowledged by the Examiner, the Moscow patent does *not* specifically teach the use of a vector comprising FPGS nor the enhancement of cytotoxic sensitivity to an anti-folate drug, such as MTX, when FPGS is expressed in neoplastic cells at a level higher than the endogenous FPGS level of said neoplastic cells. In fact, as stated above, in the Moscow patent, RFC activity is being restored in order to re-establish MTX sensitivity. This is in contrast to the present case, wherein a totally different gene, FPGS (that is still expressed at an endogenous level by the neoplastic cell) is being expressed at a level higher than the endogenous level in order to enhance MTX activity (rather than restore it).

As discussed below, Roy, Kim, and Garrow do not remedy these fundamental deficiencies in the Moscow patent.

Roy teaches that L1210 cell variants which express either decreased or increased levels of FPGS (compared with parental lines) display corresponding differences in resistance to folate analogues. According to the Examiner, Roy teaches that, *in vitro*, L1210 tumor cells resistant to MTX have a decrease in the rate of FPGS mRNA transcript formation, resulting in lower FPGS activity (page 6907, col.2, first full paragraph).

Kim teaches that mutant Chinese hamster ovary (CHO) cells, lacking FPGS activity, exhibit increased sensitivity to pulses of MTX in cell culture after being transfected with an FPGS expression cassette. Thus, Kim teaches that FPGS cDNA transfection restored cytotoxic sensitivity of FPGS-deficient CHO cells to methotrexate. Kim does not teach the transformation or transfection of neoplastic cells. In addition, Kim does not teach the transformation or transfection of cells which have some endogenous FPGS activity (like neoplastic cells), nor show that such transformation or transfection with an FPGS gene can enhance the neoplastic cell's cytotoxic sensitivity to an anti-folate drug.

Garrow teaches the cloning of a human FPGS. Garrow teaches that transfecting the cloned FPGS into mutant CHO cells lacking FPGS activity restored the ability of the transfected mutant cells to grow in culture in the absence of purines and thymidine. Garrow does not teach the delivery of a vector comprising a nucleotide molecule that encodes an FPGS into *neoplastic cells*, nor does Garrow teach the treatment of cells expressing FPGS with an antifolate drug. Further, Garrow doesn't teach or suggest that transformation or transfection of neoplastic cells with an FPGS gene can enhance the neoplastic cell's cytotoxic sensitivity to an anti-folate drug.

Rejection of claimed subject matter as obvious under 35 U.S.C. § 103 in view of a combination of references requires (1) consideration of whether prior art would have

suggested to those of ordinary skill in the art that they should make the claimed composition or carry out the claimed process, and (2) whether the prior art would also have revealed that such a person would have reasonable expectation of success; both suggestion and reasonable expectation of success must be found in the prior art, not in Applicant's disclosure. *See, In re Vaeck*, 20 U.S.P.Q.2d 1438, 1442 (Fed. Cir. 1991). Further, all claim limitations must be taught or suggested by the prior art. *In re Royka*, 180 U.S.P.Q. 580 (CCPA 1974).

Applicants maintain that the Examiner has not established a *prima facie* case of obviousness because he has not pointed to anything, in the cited references or in the body of knowledge generally possessed by those skilled in the art, that would suggest the modification or combination of the references necessary to arrive at Applicants' claimed invention. Although it might have been obvious for the skilled artisan to try and see if cytotoxic sensitivity of neoplastic cells to anti-folate drugs could be enhanced by introducing an FPGS gene, such does not give rise to a *prima facie* case of obviousness. *See, In re Geiger*, 2 U.S.P.Q.2d 1276 (Fed. Cir. 1987) and *In re Fine*, 5 U.S.P.Q.2d 1596 (Fed. Cir. 1988).

Applicants presently pending claims are not directed to the restoration of cytotoxic sensitivity to neoplastic cells that are resistant to methotrexate or other folate analogues. Rather, the present claims recite: "A method of enhancing the cytotoxic sensitivity of neoplastic cells to an antifolate drug, said method comprising:

(a) delivering directly to said neoplastic cells a vector, said vector comprising a DNA sequence encoding folylpolyglutamyl synthetase (FPGS), operably linked to a promoter, *wherein said FPGS is expressed in said neoplastic cells at a level higher than the endogenous FPGS level of said neoplastic cells*;

(b) treating the neoplastic cells in step (a) with an antifolate drug that is polyglutamated by said FPGS; and

(c) *enhancing the cytotoxic sensitivity of said neoplastic cell to said antifolate drug."* (Emphasis added).

In rationalizing the rejection, the Examiner has relied on the theory in Kim that "[l]owered FPGS activity may be a general mechanism by which cells can become resistant to a wide range of antifolates." This theory, however, does not provide the requisite motivation to modify or combine the cited references in order to arrive at the presently claimed invention, especially when the Moscow patent "teaches away" from using genes other than RFC. That is, the Moscow patent acknowledges that several different genes may be involved in the development of MTX-resistance (including decreased RFC, overexpression of dihydrofolate reductase, alteration of dihydrofolate reluctance affinity for MTX, decreased FPGS, and decreased thymidylate synthase levels), but that "decreased MTX uptake is the principal characteristic in many MTX-resistant cell-lines." Moscow Patent, col. 1, 2nd paragraph. Thus, the Moscow patent teaches how to improve the ability to transport MTX into cells (*i.e.*, uptake) by increasing the expression of RFC, rather than exploiting any other gene involved in folate metabolism. Clearly, this can be viewed as "teaching away" from using any other gene (besides RFC) since the inventors of the Moscow patent are teaching how to increase MTX uptake (by increasing RFC expression) in order to improve MTX resistance. They do not teach or suggest up- or down- regulating any other gene involved in folate metabolism.

Applicants note that the claims are not dependent on any particular mechanism of action, nor do they necessarily require that the neoplastic cells be MTX resistant. The point

of novelty of the currently claimed invention is the teaching and demonstration that, in the context of vector-mediated gene therapy, the elevation of FPGS activity beyond the endogenous level characteristic of a particular tumor cell, will augment their cytotoxic sensitivity.

As discussed above, Kim shows that vector-mediated transfection of FPGS cDNA can restore FPGS activity and reintroduce cytotoxic sensitivity into variant CHO cells that express *no endogenous* FPGS activity. In contrast, the claimed invention recites that the FPGS is transferred to neoplastic cells which have *some* endogenous FPGS activity. Applicants have shown that elevation of FPGS activity via vector-mediated gene therapy, *beyond the endogenous level characteristic of most tumor cells*, will augment their cytotoxic sensitivity. That is, the issue of whether tumor cells, already expressing FPGS, can be imbued with enhanced antifolate sensitivity after FPGS gene delivery has not been previously addressed by any of the cited art, taken alone or in combination. The limitations of the claimed method have not been met.

Applicants contend that the Examiner has not provided a sufficient explanation as to why a person skilled in the art would have been motivated to modify the teachings of Kim or Garrow such that the cloned FPGS gene is delivered, not to a mutant Chinese hamster ovary cell, but to a *neoplastic cell.*

In each cited reference, the gene being transfected is not present in the cell being studied. The cells are either FPGS deficient or RFC deficient. None of the cited references teach or suggest that FPGS can successfully be transferred to neoplastic cells which display *some* endogenous level of FPGS activity. Applicants have shown that elevation of FPGS activity via vector-mediated gene therapy, *beyond the endogenous level characteristic of*

most tumor cells, will augment their cytotoxic sensitivity. That is, the issue of whether tumor cells, already expressing FPGS, can be imbued with enhanced antifolate sensitivity after FPGS gene delivery has not been previously addressed by any of the cited art, taken alone or in combination. The limitations of the claimed method have not been met.

Applicants direct the Examiner's attention to page 40, lines 16-27, of the specification, where the teachings of Roy and Kim were discussed and distinguished from the present invention. The Applicants stated:

> It has been shown that downregulation of a tumor's FPGS activity via mutation leads to antifolate resistance (Pizzorno, G., *et al., Cancer Research 48*:2149 (1988); Roy, K., *et al., Journal of Biological Chemistry 270*:26918-26922 (1995); Roy, K., *et al., Journal of Biological Chemistry 272*:6903-6908 (1997); Takemura, Y., *et al., British Journal of Cancer 75 (suppl. 1)*:31 (1997)), and that transfection of mutant CHO cells lacking FPGS activity with a plasmid bearing the FPGS cDNA enhances their susceptibility to MTX pulses (Kim, J.S., *et al., Journal of Biological Chemistry 268*:21680-21685 (1993)). It was unclear, however, whether increasing the FPGS expression of a tumor cell line **already displaying intermediate FPGS enzyme activity** would enhance the cell line's MTX susceptibility. Emphasis added.

Since there is no motivation to modify or combine the cited references in order to arrive at the claimed invention, especially in view of the Moscow patent "teaching away" from using other known genes involved in folate metabolism, a *prima facie* case of obviousness has not been established. Accordingly, Applicants respectfully request that the rejection of the claims under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

Next, the Examiner rejects claims 14 and 28-29 under 35 U.S.C. § 103(a), because, according to the Examiner, these claims are unpatentable over the Moscow patent, in view of Roy, Kim, and Garrow, as applied to claims 14-19, 22, 25-28, 31 and 32 above, and

further in view of Pawelek *et al.*, *Cancer Research* 57:4537-4544 (1997)("Pawelek"). *See,* Paper No. 13, pages 25-26. Applicants respectfully traverse the rejection.

The teachings of all references, except Pawelek, are discussed and distinguished above. Pawelek teaches the use of an attenuated *Salmonella* as an anticancer vector for gene delivery into tumor cells. The Examiner contends that it would have been obvious for a skilled artisan to modify the combined teachings of Moscow, Roy, Kim, and Garrow for delivering a DNA sequence encoding human FPGS into tumor cells resistant to methotrexate and other folate analogues by using an attenuated *Salmonella* as an anticancer gene delivery vector, as taught by Pawelek.

Applicants respectfully submit that Pawelek does not remedy any of the fundamental defects of the prior rejection, see *supra*. Accordingly, this rejection is improper and should be withdrawn.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite allowance of this application, the Examiner is invited to telephone the undersigned directly at (202) 772-8637. Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Live bacteria as anticancer agents and tumor-selective protein delivery vectors

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The development of novel cancer therapies that are selective for cancer cells with limited toxicity to normal tissues is a challenge for oncology researchers. Microorganisms, such as viruses with selectivity for tumor cells or tumor micro-environments, have been investigated as potential arsenals for decades. Genetically-modified, non-pathogenic bacteria have begun to emerge as potential antitumor agents, either to provide direct tumoricidal effects or to deliver tumoricidal molecules. Attenuated Salmonella, Clostridium and Bifidobacterium are capable of multiplying selectively in tumors and inhibiting their growth, representing a new approach for cancer treatment. Because of their selectivity for tumor tissues, these bacteria would also be ideal vectors for delivering therapeutic proteins to tumors. VNP20009, an attenuated strain of Salmonella typhimurium, and its derivative, TAPET-CD, which expresses an Escherichia coli cytosine deaminase (CD), are particularly promising, and are currently undergoing phase I clinical trials in cancer patients.

Keywords Bifidobacterium, cancer therapy, *Clostridium*, gene therapy, *Salmonella*, vector

Abbreviations

BCG	Bacille *Calmette-Guerin*
CD	Cytosine deaminase
GFP	Green fluorescent protein
LPS	Lipopolysaccharide

Introduction

It has long been known that tumor regression occasionally occurs in patients with bacterial infections [1]. An infection arising from a tumor may be the first clinical manifestation of neoplastic disease [2]. There are many clinical reports of patients developing infected tumors, frequently with *Salmonella* [2-4]. The use of bacterial products for cancer treatment dates back to the early 1900s. William B Coley, who was then a surgeon at Memorial Hospital, now Memorial Sloan-Kettering Cancer Center, NY, USA, observed that patients with sarcoma responded better after surgery if they developed severe post-operative infections. Coley later developed a regimen involving the use of bacterial cell wall components for the treatment of cancer [5]. Based on findings in the last three decades, it is now clear that some of the antitumor activities induced by bacterial products, such as bacterial cell wall components, may be produced by cytokines released from the host immune system [6,7]. Bacille *Calmette-Guerin* (BCG), a live, attenuated mycobacterium has been used for the treatment of superficial bladder carcinoma. In patients with superficial bladder cancer, intravesically-administered BCG achieves complete remission in > 60% of patients [8,9]. The mechanism of action of BCG is unknown, although evidence suggests that induction of an antitumor immune response may be involved.

In the last several years, it has been reported that some microorganisms display selective replication in tumor cells or preferential accumulation in the tumor micro-environment. The characteristic of tumor-preferential accumulation offers great potential to amplify the therapeutic effects of microorganisms, while sparing normal tissues from toxicity. Much of the current research has focused on viruses, including vaccinia virus, Newcastle disease virus, reovirus and adenovirus with an E1a deletion, which are intended to achieve selective replication and killing of tumor cells. Observations in tumor models with bacteria date back 30 years and indicate that some bacterial species also preferentially replicate and accumulate within tumors. Bacteria possess certain features that may be advantageous in the treatment of cancer, such as motility, capacity to simultaneously carry and express multiple therapeutic proteins, and elimination by antibiotics. In this review, we shall highlight some recent findings on the use of live bacteria as antitumor agents and vectors for delivering therapeutic proteins into tumors.

Salmonella

Although wild-type *Salmonella typhimurium* is tolerated under conditions of localized infection, the major hurdle for the clinical use of *S typhimurium* in humans is its ability to induce cytokine-mediated septic shock. Salmonellosis usually occurs when a host ingests the organism via contaminated food or water. The bacteria invade the intestinal epithelium, enter the lymphatic system, and ultimately lodge in the spleen and liver. *Salmonella* infection is usually self-limiting, but if untreated, can be fatal. In order to safely use *Salmonella* clinically, either as a vaccine or an antitumor agent, the bacteria must be attenuated. Attenuated *Salmonella typhi* and *S typhimurium* have been explored for use as vaccines for many years [10,11]. Ty21a, a galE mutant of *S typhi* [12], has been approved by the US FDA as an oral vaccine for the prevention of typhoid fever. Attenuated *S typhimurium* has been used in animal models as a carrier of heterologous antigens for the prevention of viral and bacterial infections [13,16].

The genetics of *S typhimurium* are well characterized and, consequently, a variety of avirulent mutants can be generated by recombinant DNA technology. Auxotrophic mutants are the most well-known attenuated forms of *S typhimurium*. Using transposon Tn10, Stocker and his colleagues generated an aroA mutant, which was highly attenuated in mice and served as an excellent oral vaccine against challenge with the parental wild-type strain [12,17]. Several sets of genes in the bacterial genome are termed 'global regulators', which coordinate the expression of genes

under certain conditions. Two of these genes, *cya* and *crp*, discovered by Curtiss and his colleagues [18], encode proteins involved in the regulation of cyclic AMP levels in bacteria. *S typhimurium* mutants harboring *cya* and *crp* mutations are highly attenuated in mice. X3985, a *S typhimurium* mutant with *cya* and *crp* deletions, has been approved by the US Department of Agriculture as a vaccine against *Salmonella* infections in chickens. *Salmonella* mutants with deletions in the virulence-related genes *phoP/phoQ*, used as vectors to express heterologous proteins, have been evaluated in humans as vaccines [16,19,20].

In addition to its wide use in vaccines, attenuated *S typhimurium* has been evaluated as an anticancer agent. Pawelek *et al* [21••] demonstrated that attenuated *Salmonella* accumulates preferentially in syngeneic tumors and human tumor xenografts in murine models following systemic administration. In comparison studies of normal and tumor tissues, engineered *Salmonella* exhibit tumor:normal ratios of usually > 1000:1. The tumor-targeted strains of *Salmonella* also exhibit efficacy against subcutaneously implanted B16F10 melanoma. Using an attenuated strain of *S typhimurium* (SL3235) injected either intralesionally or intraperitoneally, Eisenstein *et al* [22] demonstrated growth inhibition of a murine plasmacytoma implanted subcutaneously in mice. Strain SL3235 is an aroA mutant, used extensively in vaccine development for treating Salmonellosis and other diseases. Although the mechanisms of action for *Salmonella*-induced tumor growth retardation are unknown, the authors speculate that the induction of nitric oxide-producing macrophages inside tumors may be the cause. A *cya/crp* mutant of *S typhimurium*, X4550, has been engineered to express interleukin-2 for the treatment of liver cancer in preclinical models [23•,24]. Since *S typhimurium* naturally colonizes in liver, the authors believed that attenuated *S typhimurium* could be used to deliver cytokines locally to liver and have an effect on hepatic metastases.

Bacteremia from Gram-negative bacteria can cause septic shock and death, due to an acute immune response stimulated by lipid A, the terminal moiety of lipopolysaccharide (LPS). Low *et al* [25••] have generated an attenuated *S typhimurium* mutant (YS1629) by deleting the *msbB* gene that encodes the enzyme for myristoylation of lipid A, and *purI* genes in strain YS72. They demonstrated that LPS derived from strain YS1629 does not induce TNFα production in human monocytes. Live YS1629 has a reduced capacity for inducing TNFα *in vivo*, following administration to mice and pigs. Low and his colleagues have also shown that YS1629 retains the ability to suppress the growth of B16 melanoma implanted subcutaneously into mice. VNP20009, a tetracycline-sensitive clone with deletions in both *msbB* and *purI* genes, exhibits characteristics similar to strain YS1629 [26].

The antitumor activity of VNP20009 has been evaluated in a tumor panel consisting of murine transplantable tumors and human tumor xenografts. VNP20009, administered as a single bolus intravenous injection at 1×10^4 to 1×10^5 colony forming unit (cfu)/mouse, retards the growth of subcutaneously implanted B16-F10 melanoma. The antitumor effect of VNP20009 is as good as or better than many currently used anticancer agents, such as cyclophosphamide and cisplatin. The antitumor activity of VNP20009 may not require functional T-cells, B-cells or NK cells, as suggested by its ability to induce the same activity in athymic nude, SCID and beige mice [27]. VNP20009 accumulates preferentially in tumors as compared to normal tissues. For example, VNP20009 was detected in subcutaneously implanted B16-F10 melanoma at levels as high as 2.0×10^9 cfu/g tumor, whereas the level of VNP20009 in liver was approximately 10,000-fold lower. This selectivity was observed in other subcutaneously implanted murine tumors, such as M109 lung carcinoma, and the human tumor xenografts Lox, C8186, DLD1, SW620, HCT116, HTB177, DU145, MDA-MB-231 and Caki [28••].

The mechanism for the specificity of *Salmonella* for tumors is unclear. In addition to the bacterial factors necessary for infection and penetration of tumors, several tumor-related factors are likely necessary to create a permissive environment for unchecked bacterial replication in tumors. These factors include: (i) nutrients provided by rapidly-growing and necrotic tumor cells; (ii) hypoxia, which not only allows the growth of facultative anaerobes, such as *Salmonella*, but also impairs macrophages and neutrophils, a primary defense against microbial pathogens; and (iii) irregular vasculature and positive pressure inside tumors, which block infiltration of antibodies and serum complement that lyse *Salmonella* [29,30]. Thus, *Salmonella* finds a safe haven within tumors where they multiply freely and virtually unchecked. *Salmonella* replicate to extraordinarily high levels within tumor tissue, reaching levels of $> 10^9$ cfu/g tumor, which are about 1000- to 10,000-fold greater than in normal tissues.

Salmonella possesses several characteristics that make it an excellent vector to preferentially deliver therapeutic proteins to tumors. These characteristics include: (i) motility for uniform penetration within tumors; (ii) expression of multiple proteins; (iii) production of proteins at high levels without transferring genes into mammalian cells; (iv) tropism for specific tissues, or expressed ligands that bind specific tissues; and (v) availability of avirulent mutants with minimal toxicity. Pawelek *et al* [21••] have expressed the prodrug-converting enzyme HSV-thymidine kinase (TK) in a purine auxotrophe and demonstrated enhanced antitumor activity upon the addition of ganciclovir, the corresponding prodrug. Tjuvajev *et al* [31•] have expressed HSV-TK in VNP20009 and demonstrated its selective accumulation in subcutaneously implanted murine colon 38 tumors. Using radiolabeled FIAU (a substrate for TK), the authors documented the selective accumulation of radioactivity in tumors by VNP20009-TK, which supports the utility of VNP20009-TK/FIAU as a tumor-specific imaging agent. Zheng *et al* [28••] have expressed green fluorescent protein (GFP) and cytosine deaminase (CD) in VNP20009. After injecting CD-expressing bacteria intravenously into tumor-bearing mice, the authors detected bacteria, which replicated and accumulated in tumors at levels as high as 10^9 cfu/g tissue after 4 days. In contrast, bacterial levels in normal tissues, such as liver, were usually lower than 10^6 cfu/g. CD expressed in attenuated *Salmonella* is functional, as shown by the detection of 5-fluorouracil (5FU) in tumors after injecting 5-fluorocytosine (5FC)

intraperitoneally into tumor-bearing mice. TAPET-CD, when used together with 5FC, caused tumor growth inhibition in mice bearing colon tumor xenografts [32]. These results suggest that avirulent *Salmonella* expressing prodrug-converting enzymes could be useful for converting non-toxic prodrugs to toxic metabolites in tumors to achieve antitumor effects.

Various therapeutic proteins, including TNFα, endostatin and platelet factor 4 fragment, have been cloned and expressed in VNP20009 [33,34]. Yuhua *et al* [35••] have cloned hIL-12, hGM-CSF, mIL-12 and mGM-CSF, under the control of a cytomegalovirus (CMV) promoter, into SL3261, an auxotrophic *S typhimurium*. The transformants were administered orally to mice, and soluble cytokines were detected in sera of mice receiving the transformants. A significant accumulation of GFP in tumors was detected. Yuhua *et al* also found that oral administration of *Salmonella* expressing either mGM-CSF or mGM-CSF plus mIL12 caused tumor regression in mice bearing Lewis lung carcinomas. The authors further demonstrated that these cytokines produced by the transformed *Salmonella* increase cytotoxic T-cells in peripheral blood. Because the expression of these cytokine genes was under the control of a eukaryotic promoter, the transformed *Salmonella* must be taken up by mammalian cells to produce functional proteins. Although *Salmonella* is classified as an intracellular pathogen, it is unclear whether the release of plasmid DNA from bacteria into mammalian cells is efficient.

Lee *et al* [36] have conducted preclinical toxicology of TAPET-CD in monkeys and rats, and showed that it is relatively safe at doses producing antitumor effects in rodents [32]. Cynomolgus monkeys were treated with an intravenous injection of TAPET-CD at 1×10^9 and 1×10^{10} cfu/monkey on day 1, and an oral dosing of 5FC, twice daily at 0, 250, 500, 1000 and 1500 mg/kg on days 4 to 17. TAPET-CD at 1×10^9 or 1×10^{10} cfu/monkey was well tolerated, with only occasional mild clinical signs (vomiting, inappetence, abnormal stools), increased hepatic enzyme function values and splenic enlargement. All these adverse effects disappeared within 1 week after dosing ended. TAPET-CD/5FC combination had a maximum tolerated dose of 1×10^{10} cfu/monkey for TAPET-CD and 500 mg/kg for 5FC in monkeys. TAPET-CD plus 5FC is currently being evaluated in cancer patients [37].

Clostridium

It was shown 40 years ago that certain *Clostridium* species are capable of replicating extensively in tumors, but not in normal tissues. *Clostridium*, an obligate anaerobic bacterium, selectively germinated and grew in hypoxic regions of solid tumors after intravenous injection into tumor-bearing mice. These hypoxic regions are characteristic of solid tumors in rodents and humans [38]. Because of its strict anaerobic requirement, *Clostridium* does not grow in normal tissues where oxygen is abundant. Parker *et al* [39] discovered marked lysis of tumor tissues in mice receiving an intratumoral injection of *Clostridium histolyticum* spores. The same phenomenon was observed in mice injected intravenously with spores of *Clostridium sporogenes*. The bacteria germinated and grew in tumors, causing partial tumor lysis [40]. In addition, *Clostridium* was detected only in tumors and not in normal tissues of mice receiving an intravenous injection of bacteria [41].

Because of the intriguing results in preclinical models, the M-55 strain of *Clostridium butyricum* was tested in humans for the treatment of cancer. Most patients had no objective evidence of regression or change in tumor size, but several patients developed abscesses within large tumor masses that were shown to contain the injected organism. Oncolytic activity was evident in three of five patients, occurring only in large tumors [42••]. The authors concluded that oncolysis induced by *Clostridium* appeared to be related to the physiological and biochemical characteristics of large tumor masses (anaerobiosis), rather than to the qualitative differences between normal and tumor cells. They suggested that *Clostridium* would have very limited, if any, use for the treatment of cancer, because of its inefficiency for lysing small tumors and its potential toxicity.

With advances in molecular biology and gene therapy, investigators are again exploring anaerobic *Clostridium* to deliver cytotoxic peptides and prodrug-converting enzymes specifically to tumors in animal models [43•,44-46]. Brown and co-workers [43•,45] have expressed CD in *Clostridium acetobutylicum* and demonstrated a selective delivery of the active exogenous enzyme into tumors. Lambin *et al* recently demonstrated that CD can be successfully cloned and expressed in the same strain of *Clostridium*, and CD expression was enhanced significantly by the vascular targeting agent combretastatin A-4 phosphate (OXiGENE Inc) [47]. The enhancement may be due to the enlargement of the necrotic area in tumors. Nuyts *et al* [48,49] demonstrated that functional TNFα can be cloned and expressed in *C acetobutylicum*. Most recently, a study by Vogelstein *et al* [50] reported the utility of *Clostridium novyi* in cancer therapy, and tumor regression was observed with a combination of chemotherapy and bacterial therapy in preclinical models. The authors noted that *C novyi* was useful for large tumors with necrotic areas, but was less efficacious for micrometastases.

The preclinical and clinical studies with *Clostridium*, a spore-forming Gram-positive bacteria, identified several obstacles to the development of preferentially replicating bacteria for the treatment of cancer. Successful clinical candidates should: (i) infect and multiply within both small and large metastatic lesions; (ii) be sufficiently attenuated to avoid induction of severe systemic inflammatory responses, which could lead to excessive toxicity; and (iii) be sensitive to common antibiotics to provide a margin of safety.

Bifidobacterium

Bifidobacterium is a genus of non-pathogenic, Gram-positive, anaerobic lactic acid bacteria. It is a normal constituent of the alimentary tract of humans, as well as animals, including chickens, rabbits, pigs, cows and sheep.

Like *Clostridium*, the basis for *Bifidobacterium* selective colonization of tumors is apparently due to their requirement for anaerobiasis, a condition common in larger tumors due to irregular angiogensis and necrosis. Unlike *Clostridium*, *Bifidobacterium* does not form antibiotic-resistant spores, a potential safety advantage for a live, replication-

competent anticancer agent. Although there is considerable safety experience with the use of *Bifidobacterium* in humans when taken orally to assist in stabilizing intestinal flora, there is no known experience with intravenous administration to humans. Cell wall extracts have been used as immunomodulators, similarly to BCG [51,52].

Bifidobacterium bifidum was first used by Kimura *et al* [53] for studies of tumor colonization. These authors primarily used Ehrlich ascites tumors implanted in the thigh muscle of DDD-H-2^s mice and staged to 4 weeks. A suspension of lyophilized bacteria at 6×10^6 cfu was injected systemically into the tail vein. Proliferation of the bacteria was assisted by daily intraperitoneal injections of lactulose, a sugar substrate metabolized by bacteria, but not by mammalian cells. The addition of lactulose increased colonization of bacteria within tumors by 1000-fold compared to controls. Biodistribution of bacteria was determined, and counts showed highly specific accumulation of bacteria in tumors, with virtually no bacteria in other organs after 96 h. The amount of bacteria within tumors at 1 h was 1×10^2 cfu, rising to 1×10^6 cfu by day 7. With an injection of 5×10^6 cfu/mouse, tumor targeting occurred best with tumors of 15 mm in diameter or greater. At the same dose, targeting tumors smaller than 15 mm resulted in a significant drop in the percentage of tumors targeted. However, higher doses allowed colonization of similarly-sized smaller tumors. No antitumor effects or prolongation of survival was found. These studies suggested the potential use of *Bifidobacterium* as a tumor-specific delivery vector. Although the number of bacteria in tumors was low compared to that achieved by *Salmonella* and *Clostridium*, there could be a potential use for *Bifidobacterium* to deliver very potent cytotoxic peptides to tumors.

Subsequent work on *Bifidobacterium* was carried out by Fujimori *et al* [54] using *Bifidobacterium longum*. The authors used C57BL/6 mice implanted with B16-F10 melanoma or Lewis lung carcinoma staged to 2 weeks. The ability of the bacterium to carry a plasmid bearing a spectinomycin marker to the tumor was assessed; approximately 1×10^6 spectinomycin-resistant colonies were obtained for both tumor types. These data indicate that *Bifidobacterium* could be used to deliver a plasmid-encoded antitumor effector gene, similarly to *Salmonella* and *Clostridia*, studies on which have already been reported. Lactulose supplementation was also used in these studies. Further work by the same group [55•] showed that *Bifidobacterium* tumor targeting also occurs in carcinogen-induced mammary tumors in rats (carcinogen-induced tumors are believed to be more representative of naturally occurring tumors). No antitumor activity, however, was demonstrated.

Conclusions

The use of live, attenuated bacteria as antitumor agents and therapeutic protein delivery vectors has shown great promise in the last several years. VNP20009 and TAPET-CD are being evaluated in human clinical trials, and results from these studies will undoubtedly provide a better understanding of the ultimate likelihood of success for this approach. It is also likely that other live, attenuated bacteria, such as *Clostridia* and *Bifidobacterium*, will be evaluated in human clinical trials in the future. One advantage to using *Salmonella* instead of *Clostridium* or *Bifidobacterium* is its ability to grow in both aerobic and anaerobic conditions, indicating its usefulness against small tumors.

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• of special interest

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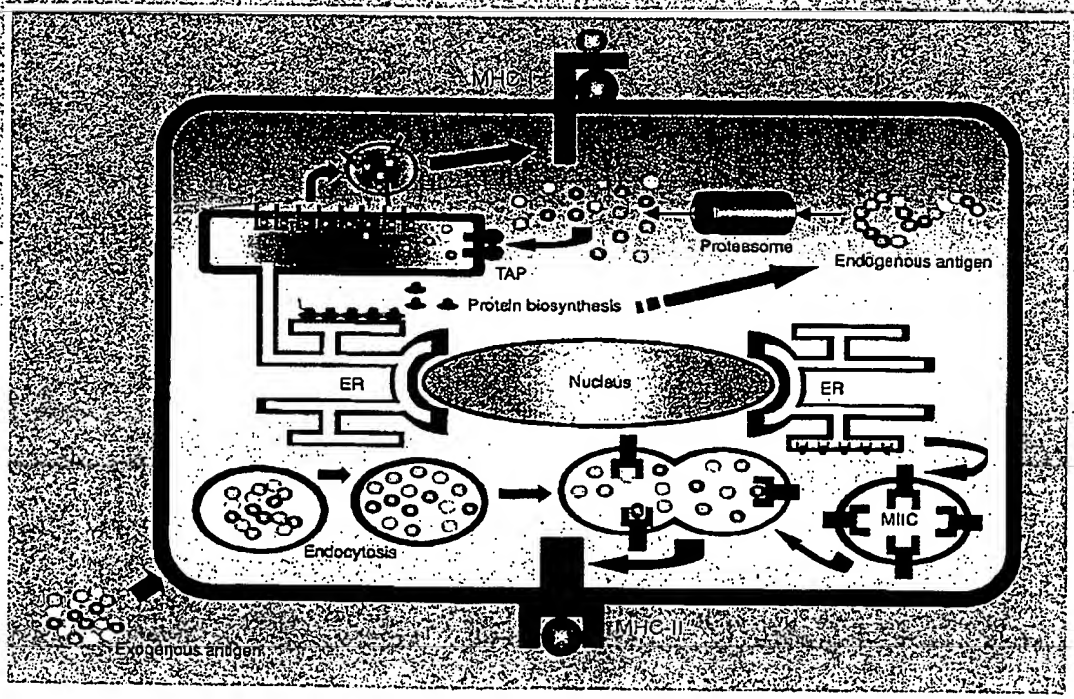

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